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Date: May 28, 2003

Arun Chakrabarti

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From: Pamela Sherwood

Total number of pages, including this cover sheet: 2

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Attached please find a PTOL-413A Interview Request Form

PTOL-413A (05-03)
Approved for use through xx/xx/xxxx OMB 0651-00xx
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

Applicant Initiated Interview Request Form

Application No.: 10/027807 First Named Applicant: Li Gan
Examiner: A. Chakrabarti Art Unit: 1634 Status of Application: Final OA

Tentative Participants:
(1) Arun Chakrabarti (2) Pamela Sherwood
(3) Rebecca Taylor (4)

Proposed Date of Interview: May 29, 2003 **Proposed Time:** 10:00 (AM/PM) PACIFIC TIME

Type of Interview Requested:
(1) [X] Telephonic (2) [ ] Personal (3) [ ] Video Conference

Exhibit To Be Shown or Demonstrated: [Y] YES [ ] NO
If yes, provide brief description: Summary in art defining differences between RNAi and antisense; and induction of interferon response by RNAi in mammalian cells.

Issues To Be Discussed

Issues (Rej., Obj., etc)	Claims/ Fig. #s	Prior Art	Discussed	Agreed	Not Agreed
(1) 103(a)	14-15, 17, 23-25 30, 51, 34-36, 43 52-53	Leptin, Der	[ ]	[ ]	[ ]
(2) 103(a)	19-20	Leptin, Der Petryshyn	[ ]	[ ]	[ ]
(3)			[ ]	[ ]	[ ]
(4)			[ ]	[ ]	[ ]

[ ] Continuation Sheet Attached

Brief Description of Arguments to be Presented:
Invertebrate cells lack interferon response and therefore cannot predict the results of RNAi in mammalian cells, which have an interferon response. RNAi and antisense RNA involve different molecules and mechanisms; one does not obviate the other.

An interview was conducted on the above-identified application on _____.

NOTE:
This form should be completed by applicant and submitted to the examiner in advance of the interview (see MPEP § 713.01).
This application will not be delayed from issue because of applicant's failure to submit a written record of this interview. Therefore, applicant is advised to file a statement of the substance of this interview (37 CFR 1.133(b)) as soon as possible.

Pamela Sherwood
(Applicant/Applicant's Representative Signature) (Examiner/SPE Signature)

CERTIFICATE OF TRANSMISSION			
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Date: May 28, 2003

To: **Please deliver to Examiner Arun K. Chakrabarti**
Art Unit 1634, USPTO

Facsimile No.: (703) 305-3014

From: Susan M. Alessi assisting
Pamela J. Sherwood, Ph.D., Reg. No. 36,677

Re: U.S. Patent Application No. 10/027,807
Title: HIGH THROUGHPUT TRANSCRIPTOME AND FUNCTIONAL VALIDATION ANALYSIS
Inventor(s): GAN et al.
Attorney Docket No.: AGYT-013CIP

Message:

- **Transmittal (1 page)**
- **Communication (1 page)**
- **Exhibits (13 pages)**

Please find attached for your phone conference with Pam tomorrow. If there are any problems concerning the transmission for these documents please contact Susan M. Alessi at (650) 833-7714 or Via email at alessi@bozpat.com.

Thank you!

Total number of pages, including this cover sheet: 16

Please type a plus sign (+) inside this box → [+]

PTO/SB/21 (08-00)
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TRANSMITTAL FORM

(to be used for all correspondence after initial filing)

Total Number of Pages In This Submission	16

Application Number	10/027,807
Filing Date	October 19, 2001
First Named Inventor	GAN, LI
Group Art Unit	1634
Examiner Name	CHAKRABARTI, ARUN K.
Attorney Docket Number	AGYT-013CIP

ENCLOSURES (check all that apply)

- [ ] Fee Transmittal Form
  - [ ] Fee Attached
- [ ] Amendment / Reply
  - [ ] After Final
  - [ ] Affidavits/declaration(s)
- [ ] Extension of Time Request
- [ ] Express Abandonment Request
- [ ] Information Disclosure Statement
- [ ] Certified Copy of Priority Documents
- [ ] Response to Missing Parts/ Incomplete Application
  - [ ] Response to Missing Parts under 37 CFR 1.52 or 1.53
- [ ] Assignment Papers *(for an Application)*
- [ ] Drawing(s)
- [ ] Licensing-related Papers
- [ ] Petition
- [ ] Petition to Convert to a Provisional Application
- [ ] Power of Attorney, Revocation Change of Correspondence Address
- [ ] Terminal Disclaimer
- [ ] Request for Refund
- [ ] CD, Number of CD(s)
- [ ] After Allowance Communication to Group
- [ ] Appeal Communication to Board of Appeals and Interferences
- [ ] Appeal Communication to Group *(Appeal Notice, Brief, Reply Brief)*
- [ ] Proprietary Information
- [ ] Status Letter
- [x] Other Enclosure(s) *(please identify below)*:
  1. **Communication**
  2. **Exhibits**
  3. **Fax Cover Sheet**

Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm or Individual Name	**PAMELA J. SHERWOOD, Reg. No. 36,677**
Signature	Pamela Sherwood
Date	**May 28, 2003**

CERTIFICATE OF FACSIMILE TRANSMISSION

I hereby certify that this correspondence is being facsimile filed under 37 C.F.R. §§ 1.6(d) and 1.8(a)(1)(b) addressed to: Commissioner for Patents Commissioner for Patents, Washington, DC 20231 on this date: May 28, 2003

Typed or printed name	Susan M. Alessi		
Signature	Susan Alessi	Date	May 28, 2003

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Communication		
Address to: Assistant Commissioner for Patents Washington, D.C. 20231	Attorney Docket No.	AGYT-013CIP
	Confirmation No.	9177
	First Named Inventor	L. Gan
	Application Number	10/027,807
	Filing Date	October 19, 2001
	Group Art Unit	1634
	Examiner Name	A. Chakrabarti
	Title: High Throughput Transcriptome and Functional Validation Analysis	

Sir:

Prior to Applicants' telephone conference with the Examiner on May 29, 2003, Applicants would like to provide the Examiner with the attached review (from Kimball's Biology Pages), which briefly summarizes the use of antisense RNA, which is a single stranded molecule complementary to an mRNA; and RNAi, which is a double stranded molecule.

Also attached is a review of RNAi, which discusses, in accordance with Applicants prior response, the effect of double-stranded RNA in triggering an interferon response in mammalian cells (see Figure 3).

If the Examiner finds that a Telephone Conference would expedite the prosecution of this application, he is invited to telephone the undersigned at the number provided.

The Commissioner is hereby authorized to charge any other fees under 37 C.F.R. §§ 1.16 and 1.17 which may be required by this paper, or to credit any overpayment, to Deposit Account No. 50-0815, order number AGYT-013CIP.

Respectfully submitted,

Date: May 28, 2003

By: _____
Pamela J. Sherwood, Ph.D.
Registration No. 36,677

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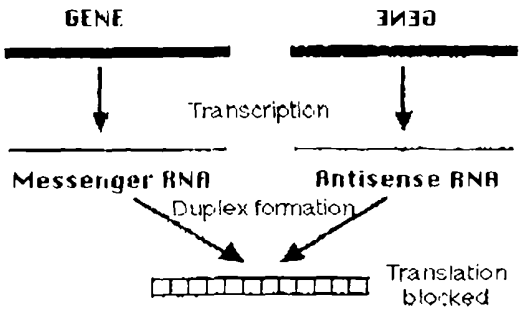
Antisense RNA



Messenger RNA (mRNA) is single-stranded. Its sequence of nucleotides is called "sense" because it results in a gene product (protein). Normally, its unpaired nucleotides are "read" by transfer RNA anticodons as the ribosome proceeds to translate the message.

5' ...A T G G C C T G G A C T T C A... 3' Sense strand of DNA
3' ...T A C C G G A C C T G A A G T... 5' Antisense strand of DNA

↓ Transcription of antisense strand

5' ...A U G G C C U G G A C U U C A... 3' mRNA

↓ Translation of mRNA

Met — Ala — Trp — Thr — Ser — Peptide

However, RNA can form duplexes just as DNA does. All that is needed is a second strand of RNA whose sequence of bases is complementary to the first strand; e.g.,

5' C A U G 3' mRNA
3' G U A C 5' Antisense RNA

The second strand is called the antisense strand because its sequence of nucleotides is the complement of message sense. When mRNA forms a duplex with a complementary antisense RNA sequence, translation is blocked. This may occur because the ribosome cannot gain access to the nucleotides in the mRNA or duplex RNA is quickly degraded by ribonucleases in the cell.

With recombinant DNA methods, synthetic genes (DNA) encoding antisense RNA molecules can be introduced into the organism.

Example: the Flavr Savr tomato. Most tomatoes that have to be shipped to market are harvested before they are ripe. Otherwise, enzymes synthesized by the tomato cause them to

spoil before they reach the customer.

Transgenic tomatoes have been constructed that carry in their genome an artificial gene (DNA) that is transcribed into an antisense RNA complementary to the mRNA for an enzyme involved in spoilage. These tomatoes make only 10% of the normal amount of the enzyme. The goal of this work was to provide supermarket tomatoes with something closer to the appearance and taste of tomatoes harvested when ripe.

Antisense RNA for human therapy. Antisense RNA that is complementary to the proto-oncogene BCL-2 is being examined as a possible therapy for certain B-cell lymphomas and leukemias. Antisense oligodeoxynucleotides (ODNs) are synthetic molecules that - because they, too, are antisense - also block mRNA translation. One has been approved for human therapy

Antisense RNA also occurs naturally. Do cells contain genes that are naturally translated into antisense RNA molecules capable of blocking the translation of other genes in the cell? Recently a few cases have been found and these seem to represent another method of regulating gene expression.

In both mice and humans, the gene for the insulin-like growth factor 2 receptor (Igf2r) that is inherited from the father synthesizes an antisense RNA that appears to block synthesis of the mRNA for Igf2r. An inherited difference in the expression of a gene depending on whether it is inherited from the mother or the father is called genomic or parental imprinting.

RNA interference (RNAi)

In testing the effects of antisense RNA, one should use sense RNA of the same coding region as a control. Surprisingly, preparations of sense RNA often turn out to be as effective an inhibitor as antisense RNA.

Why? It seems that the preparations of sense RNA often are contaminated with hybrids: **sense and antisense strands that form a double helix of double-stranded RNA (dsRNA)**. Double-stranded RNA corresponding to a particular gene is a powerful suppressant of that gene. In fact, the suppressive effect of antisense RNA probably also depends on its ability to form dsRNA (using the corresponding mRNA as a template).

The ability of dsRNA to suppress the expression of a gene corresponding to its own sequence is called RNA interference (RNAi). It is also called post-transcriptional gene silencing or PTGS.

Mechanism of RNAi. The only RNA molecules normally found in the cytoplasm of a cell are molecules of single-stranded mRNA. If the cell finds molecules of double-stranded RNA dsRNA, it uses an enzyme (the one in Drosophila has been named Dicer) to cut them into fragments containing 21-25 base pairs (~ 2 turns of a double helix).

The two strands of each fragment then separate enough to expose the antisense strand so that it can bind to the complementary sense sequence on a molecule of mRNA. This triggers cutting the mRNA in that region thus destroying its ability to be translated into a polypeptide. Because of their action, these fragments of RNA have been named "short (or small) interfering RNA" (siRNA).

RNAi has been found to operate in such diverse organisms as plants, fungi, and animals such as Drosophila, C. elegans, and even mice and the zebrafish. Such a universal cell response must have an important function. What could it be?

One possibility. The viruses of both plants and animals have a genome of dsRNA. And many other viruses of both plants and animals have an RNA genome that in the host cell is briefly converted into dsRNA. So RNAi may be a weapon to counter infections by these viruses by destroying their mRNAs and thus blocking the synthesis of essential viral proteins.

Another possibility. In C. elegans, successful development through its larval stages and on to the adult requires the presence of at least two "small temporal RNAs" ("stRNAs")- single-stranded RNA molecules containing about 22 nucleotides - thus the same size as the fragments made by the Drosophila Dicer gene. These small transcripts are generated by the cleavage of larger precursors using the C. elegans version of Dicer. They act by inhibiting translation of several messenger RNAs in the worm (by binding to a region of complementary sequence in the 3' untranslated region [3'UTR] of the mRNA). So RNA interference may be the unexpected dividend of a another basic process of controlling gene expression.

RNAi as a tool. In any case, the discovery of RNAi adds a promising tool to the toolbox of molecular biologists. Introducing dsRNA corresponding to a particular gene will knock out the cell's own expression of that gene.

RNAi : the review

Most readers of this article are not familiar with RNA interference or may even have never heard about this new powerful technology. In a few chapters, this review will guide you step-by-step towards a better understanding of what is RNA interference, how does it work, what should be done, and what should be avoided. With our help, you will discover a new fascinating world: gene suppression through small RNA molecules.

Establishing a convenient and reliable method to knock-out gene expression at the mRNA level has been the dream and nightmare of molecular biologists for the last 15 years. In efforts to generate loss-of function cells or organisms, various molecules that included, for example, antisense sequences, ribozymes, and chimeric oligonucleotides have been tested, but the design of such molecules was based on trial and error, depending on the properties of the target gene. Moreover, the desired effects were difficult to predict, and often only weak suppression achieved (Braasch and Corey, 2002).

More than a decade ago, some botanists won the jackpot unconsciously. In 1990, two teams lead respectively by Napoli and Stuitje first reported the co-suppression of an overexpressed *chalcone synthase* (CHS) in plants. When trying to create more purple petunias, they sometimes achieved an unexpected opposite result (more white petunias!). The mechanism of this curious phenomenon remained a mystery, but it was proposed that the products of degradation of the double-stranded RNA region in the CHS gene might be related to this post-transcriptional gene silencing (PTGS) (Van der Krol et al., 1990 · Jorgensen et al., 1996) (Table 1).

In the fungi *Neurospora crassa*, it was shown that an overexpressed transgene can also induce gene silencing at the post-transcriptional level, a phenomenon referred to as quelling (Romano and Macino, 1992) (Table 1).

In 1998, building on these previous studies, Andy Fire of the Carnegie Institute and Craig Mello of the University of Massachusetts for the first time demonstrated with the worm *Caenorhabditis elegans* that dsRNA (double-stranded RNA) may specifically and selectively inhibit the gene expression in an extremely efficient manner. In their experiment, the sequence of the first strand (the so-called *sense* RNA) coincides with that of the corresponding region of the target messenger RNA (mRNA). The second strand (*antisense* RNA) is complementary to this mRNA. The resulting dsRNA turned out to be far more (several orders of magnitude) efficient

than the corresponding single-stranded RNA molecules (in particular, *antisense* RNA). Fire et al., 1998 named the phenomenon RNAi for RNA *interference*. This powerful gene silencing mechanism has been shown to operate in several species among most phylogenetic phyla (Table 2).

Table 1: Post-transcriptional gene silencing mechanisms

Phylum	Species	Mechanism	Effector	Reference
Fungi	*Neurospora*	quelling	Transgenes	Cogoni and Macino, 1997.
Plants	*Arabidopsis*	PTGS	Transgenes	Elmayan et al., 1998
	Petunia			Dehio and Schell, 1994.
	Nicotiana	Transcriptional gene silencing	Transgenes, virus	Fumes et al., 1995.
Invertebrates	*C. elegans*	RNAi	dsRNA	Ketting et al., 1999.
		Transcriptional gene silencing	Transgenes	Kelly and Fire, 1998.
	Drosophila	RNAi	dsRNA	Misquitta and Paterson, 1999
			dsRNA	Fagioloni et al., 2002
		co-suppression	Transgenes	Pal-Bhadra et al., 1999.
	Paramecium	Homology-dependent silencing	Transgenes	Ruiz et al., 1998.
	Trypanosoma	RNAi	dsRNA	Wang et al., 2000
Vertebrates	*Danio rerio*	RNAi	dsRNA	Wargelius et al., 1999
	Mus musculus	RNAi	dsRNA	Wianny and Zernicka-Goetz, 2000.

Table 2: Examples of RNAi in several species

Species		References
Caenorhabditis elegans	Nematode	Fire et al., 1998; Tavernarakis et al., 2000
Danio rerio	Zebrafish	Wargelius et al., 1999
Trypanosoma brucei	Unicellular	Wang et al., 2000
Hydra magnipapillata	Cnidarian	Lohmann et al., 1999
Schmidtea mediterranea	Planarian	Sanchez and Newmark, 1999
Escherichia coli	Bacteria	Tabara et al., 2000
Neurospora crassa	Fungus	Cogoni and Macino, 1999
Drosophila melanogaster	Fruitfly	Boutros et al., 2001
Mus musculus	Mammals	Wianny and Zernicka-Goetz, 1999
Arabidopsis thaliana	Plants	Mette et al., 2001

RNAi begins when an enzyme, which Hannon and colleague Emily Bernstein discovered and named DICER, encounters dsRNA and chops it into pieces called small-interfering RNAs or siRNAs. This protein belongs to the RNase III nuclease family. A complex of proteins gathers up these RNA remains and uses their code as a guide to search out and destroy any RNAs in the cell with a matching sequence, such as target mRNA (for review see Bosher and Labouesse, 2000).

Figure 1 depicts an updated model of the RNAi phenomenon (Akashi et al., 2001; Willecke et al., 2002). In this model, the initiator event coincides with the appearance in a cell of transgenes, transposons, virus, dsRNA or aberrant single-stranded RNA. In the latter case, as described for quelling, RNA-dependent RNA Polymerase (RdRP) is responsible for the production of dsRNA. The following steps might be summarized as follows:

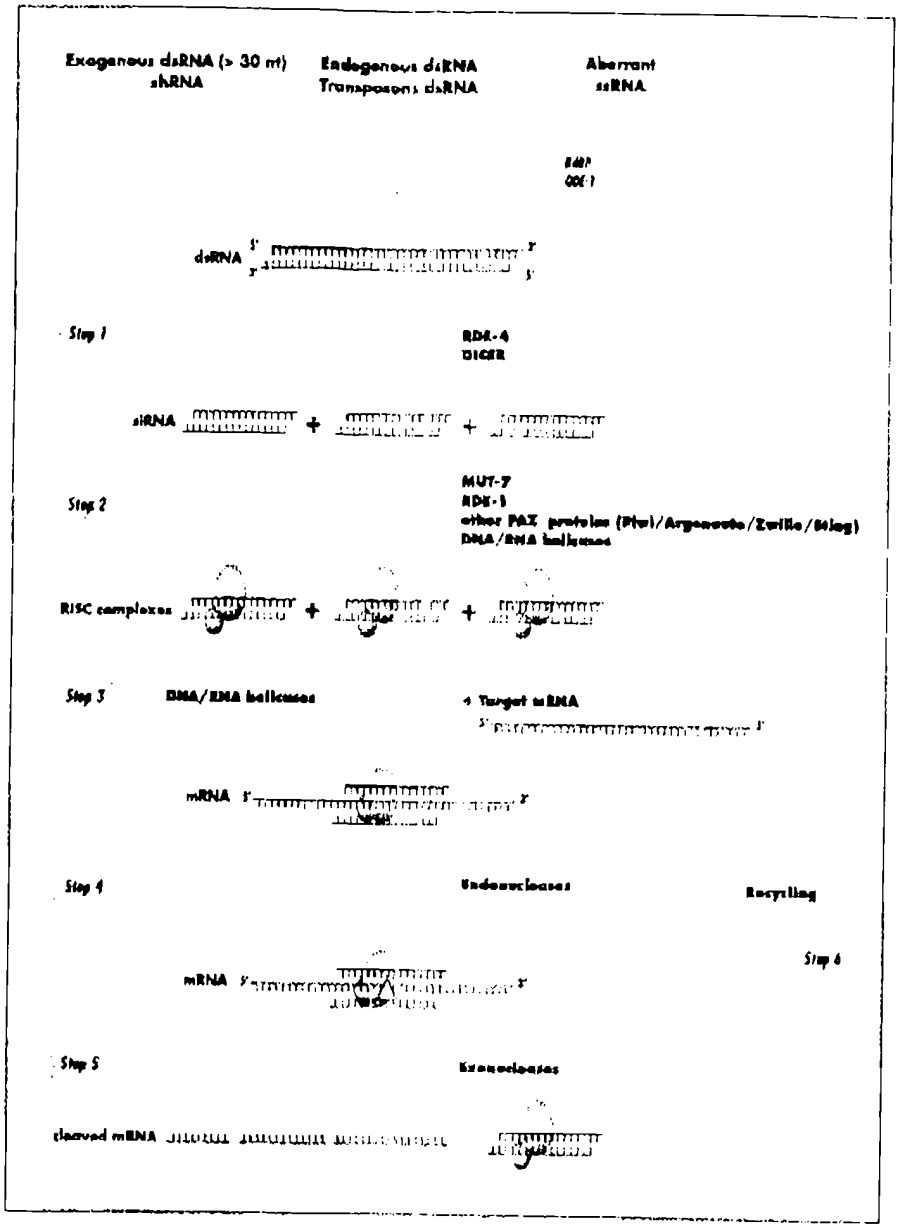


Figure 1: RNAi hypothetical model

- Step 1: dsRNA recognition and scanning process.
- Step 2: dsRNA cleavage through RNase III activity and production of siRNAs.
- Step 3: association of the siRNAs and associated factors in RISC complexes.
- Step 4: recognition of the complementary target mRNA.
- Step 5: cleavage of the target mRNA in the center of the region complementary to the siRNA (see yellow triangle).
- Step 6: degradation of the target mRNA and recycling of the RISC complex (see figure 2).

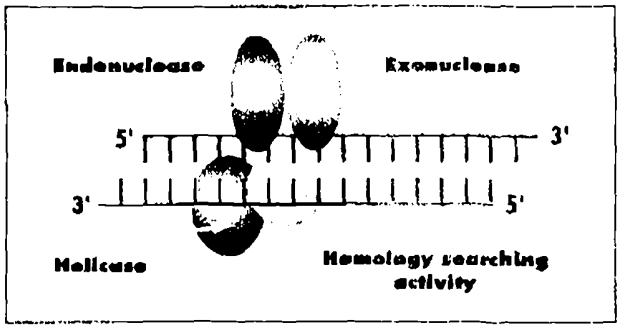


Figure 2: RISC complex hypothetical model

RNAi is extremely active in several invertebrate species. Therefore, it was highly tempting to adapt this technology to mammals. However, mammalian cells have developed various protective phenomena against viral infections that could impede the use of this approach. Indeed, the presence of extremely low levels of viral dsRNA triggers an interferon response (called "acute-phase response") and the activation of a dsRNA Responsive Protein Kinase (PKR). PKR phosphorylates and inactivates translation factor Elf2a leading to activation of the 2', 5' oligoadenylate synthetase, finally resulting in RNAse L activation. This cascade induces a global non-specific suppression of translation, which in turn triggers apoptosis (for review see Williams, 1997; Gil and Esteban, 2000).

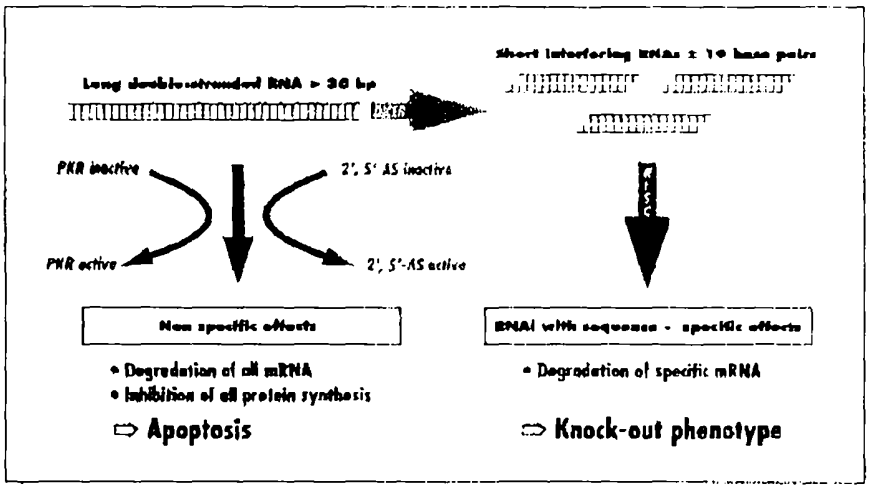


Figure 3: Non-specific and specific dsRNA silencing pathways

In 2000, a first attempt was made with dsRNA in mouse embryos. Wianny and Zernicka-Goetz have shown that injected dsRNA specifically inhibit 3 genes (MmGFP under the control of the Elongation Factor 1a, E-cadherin, and c-mos) in the mouse oocyte and early embryo. Translational arrest, and thus a PKR response, was not observed as the embryos continued to develop.

However, we had to wait another year before covering the decisive step. Procedures developed by *Ribopharma AG* (Kulmbach, Germany) (and for which a patent has been granted) first demonstrated the functionality of RNAi in

mammalian cells. *Ribopharma's* researchers reasoned that smaller dsRNA, similar to those produced by DICER, should not trigger cell death. This proved to be correct and by using short (20-24 base pairs) dsRNAs - which are called *SIRPLEX™* in *Ribopharma's* terminology – they specifically switched off genes even in human cells without initiating the acute-phase response. Thus, *SIRPLEX™* is suitable for gene target validation and therapeutic applications in many species, including humans. Similar experiments carried out later by other research groups (Elbashir et al., 2001; Caplen et al., 2001) further confirmed these results. From that date, small dsRNA, called siRNA for small-interfering RNAs, become the preferred RNAi effector in many laboratories.

Driven in part by their desire for an alternative to siRNAs, Paddison et al. (2002) tried to use small RNAs folded in hairpin structures to inhibit the function of specific genes. This work was inspired by previous studies showing that some genes in *Caenorhabditis elegans* naturally regulate other genes through RNAi by coding for hairpin-structured RNAs. Tested in a variety of normal and cancer human and mouse cell lines, short hairpin RNAs (shRNAs) are able to silence genes as efficiently as their siRNA counterparts. Moreover, shRNAs exhibits better reassociation kinetics *in vivo* than equivalent duplexes. Even more important, these authors generated transgenic cell lines engineered to synthetize shRNAs that exhibit a long-lasting shagging effect throughout cell divisions. It should be noted that Eurogentec's RNA synthesis platform represents the first commercial source of shRNAs.

Recently, another group of small RNAs (also comprised in the range of 21-25 nt) was shown to mediate downregulation of gene expression. These RNAs, known as small temporally regulated RNAs (stRNAs), have been described in *Caenorhabditis elegans* were they regulate timing of gene expression during development. It should be noted that stRNAs and siRNAs, despite obvious similarities, proceed through different modes of action (for review see Banerjee and Slack 2002). In contrast with siRNAs, 22 nt long stRNAs downregulate expression of target mRNA after translational initiation without affecting mRNA integrity. Recent studies indicate that the two stRNAs first described in nematods are the members of a huge family with hundreds of additional micro-RNAs (miRNAs) existing in metazoans (Grosshans and Slack, 2002).

Scientists have initially used RNAi in several systems, including *Caenorhabditis elegans*, Drosophila, trypanosomes, and various other invertebrates. Moreover, using this approach, several groups have recently presented the specific suppression of protein biosynthesis in different mammalian cell lines - specifically in HeLa cells - showing that RNAi is a broadly applicable method for gene silencing in vitro. Based on these results, RNAi has rapidly become a well-recognized tool for validating (identifying and assigning) gene functions. With the increasing importance of Proteomics it will rapidly gain credit. RNA interference employing short dsRNA oligonucleotides will, moreover,

permit to decipher the function of genes being only partially sequenced.

RNAi will therefore become inevitable in studies such as:

• Inhibition of gene expression at the post-transcriptional level in eucaryotic cells. In this context, RNAi is a straight-forward tool to rapidly assess gene function and reveal null phenotypes.

• Development of the RNAi technology for use in post-implantation embryos.

• The predominant economic significance of RNA interference is established by its application as a therapeutic principle. As so, RNAi may yield RNA-based drugs to treat human diseases.

In 1999, Tuschl et al. have deciphered the silencing effect of siRNAs showing that their efficiency is a function of the length of the duplex, the length of the 3'-end overhangs, and the sequence in these overhangs.

Based on this founder work, Eurogentec recommends that the target mRNA region, and hence the sequence of the siRNA duplex, should be chosen using the following guidelines :

• Since RNAi relies on the establishment of complex protein interactions, it is obvious that the mRNA target should be devoided of unrelated bound factors. In this context, both the 5' and 3' untranslated regions (UTRs) and regions close to the start codon should be avoided as they may be richer in regulatory protein binding sites.

• The sequence of the siRNA is therefore selected as follows :

  - In your mRNA sequence, select a region located 50 to 100 nt downstream of the AUG start codon.
  - In this region, search for the following sequences: AA(N19)TT or AA(N21). Calculate the G/C percentage for each sequence identified. Ideally, the G/C content is 50 % but it must less than 70 % and greater than 30 %.
  - Perform a BLAST (i.e. NCBI ESTs database) with the nucleotide sequence fitting best the previous criteria to ensure that only one gene will be inactivated.
  - Don't pay too much attention to the secondary structure of the target mRNA since it does not have a strong effect on the observed silencing effect.

The selection process is that simple and proved its efficiency in numerous studies!

In collaboration with leading authorities in the field of antisense studies, Eurogentec has selected from the literature a set of validated siRNA sequences. A comprehensive list of the corresponding target genes is given in Table 3. These genes may serve as positive controls. More information about these genes is available upon request.

Table 3: Validated siRNAs available from Eurogentec

Gene name	Species	Gene name	Species
Beta actin	Human	Keratin 18	Human
Gamma-actin	Human	Lamin A/C, B1, B2	Human
NSC21	Human	Lamarins	Human
AIR binding protein	Human	YASK	Human
UAP-2	Human	h/m Shc	Human
NuMA	Human	Cyclin B1	Xenopus laevis
cdk1	Human	Cyclin B2	Xenopus laevis
CENP-E	Human	Vimentin	Mouse
Dynein 1 heavy chain	Human	Ezrin	Mouse
Eg-5	Human	GFP	Jellyfish
Emerin	Human	CAT	Bacteria
GAS41	Human	Luciferases GL2, GL3	Firefly

Every researcher would tell it: "*The choice of the right controls makes the whole difference between a good and a bad experiment*". This adage is particularly true for RNAi studies.

Therefore, to maximize your result interpretation, the following precautions should be taken when using siRNAs:

- Always test the sense and antisense single strands in separate experiments.
- Try to use a scramble siRNA duplex. This should have the same nucleotide composition as your siRNA but lack significant sequence homology to any other gene (including yours).
- If possible, knock-down your gene with two independent siRNA duplexes to control the specificity of the silencing process.

An annealing step is necessary when working with single-stranded RNA molecules. It is critical that all handling steps be conducted under sterile, RNase-free conditions.

To anneal the RNAs, the oligos must first be quantified by UV absorption at 260 nanometers (nm). RNAs ordered from Eurogentec are always quantified with the highest accuracy. The following protocol based on Elbashir et al. (2001) is then used for annealing :

- Separately aliquot and dilute each RNA oligo to a concentration of 50 µM.
- Combine 30 µl of each RNA oligo solution and 15 µl of 5X annealing buffer. Final buffer concentration is: 100 mM potassium acetate, 30 mM HEPES-KOH pH 7.4, 2 mM magnesium acetate. Final volume is 75 µl.
- Incubate the solution for 1 minute at 90 °C, centrifuge the tube for 15 seconds, let sit for 1 hour at 37 °C, then use at ambient temperature. The solution can be stored frozen at -20 °C and freeze-thawed up to 5 times. The final concentration of siRNA duplex is usually 20 µMolar.

Despite its extreme efficiency, the selected siRNA might not work in your cell system. If so, it is advisable to check the following points :

- If no knock-out of the target gene is observed, it may be useful to analyze whether the corresponding mRNA was effectively degraded upon addition of the siRNA. Two or three days after transfection, the total RNA is extracted and subjected to further analysis. RT/PCR appears to be the method of choice since it is faster and far more sensitive than Northern blotting.
- Check for any sequencing error or polymorphism in your target gene. It has been shown that a single base mutation in the pairing region of the siRNA duplex is sufficient to abolish RNAi.
- Check that your cell line can effectively express the target mRNA.

Eurogentec's siRNAs are usually synthetized at the 0.2 or 1 µmol scale. You might receive them either highly pure (>95%) or simply crude with their protective groups still attached (> 80% purity).

By default, we propose dTdT overhangs at the 3' ends, which typically provide more reliable synthesis and stability than UU. However, you may specify any mixture of DNA and RNA bases to fit your experimental requirements.

Finally, Eurogentec proposes more than 10 chemical modifications allowing the fanciest experiments (Table 4).

RNAs are famous for their ability to form stable secondary structures. Based on this observation, Eurogentec's siRNA oligonucleotides are proposed PAGE purified, the best purification method to remove all but full-length synthesis products.

Eurogentec has been at the leading edge of the QC methods, introducing Mass Spectrometry MALDI-TOF Quality Control in 1999.

When you order one of our siRNA sets, you receive single-strand siRNAs in separate tubes either lyophilized (PAGE purified) or in solution (crude). Such conditioning may seem bothering but it has several key advantages by allowing you

- to use each single oligo as a negative control.
- to test various combinations of modified strands.

It is clear : RNAi is a powerful method. However, as any other antisense technology, this must be handled and interpreted with great care. Inevitably negative controls will represent unpleasant extra costs. In this context, Eurogentec has decided to propose affordable high-quality siRNAs so that well-controlled RNAi experiments will become feasible even in small labs.

Table 4: Modifications available for siRNA

LABELLED siRNA

Modification	Reference
5' HEX / TET	OR-0030-MX0x/TEX5
5' Fluorescein, 6-FAM	OR-0030-MX0x/FAM5
Fluorescein dT internal	OR-0030-MX0x/FLUID
3' Fluorescein, 6-FAM	OR-0030-MX0x/FAM3
5' TAMRA, Rhodamine	OR-0030-MX0x/TAMR5
3' TAMRA, Rhodamine	OR-0030-MX0x/TAMR3
5' Phosphate	OR-0030-MX0x/PO4

If you can't find your particular modification, please don't hesitate to contact us.

SEE
www.eurogentec.com
siRNA chapter

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